Ecological Immunology of mosquito-malaria interactions: Of non-natural versus natural model systems and their inferences

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SUMMARY

There has been a recent shift in the literature on mosquito/*Plasmodium* interactions with an increasingly large number of theoretical and experimental studies focusing on their population biology and evolutionary processes. Ecological immunology of mosquito-malaria interactions – the study of the mechanisms and function of mosquito immune responses to *Plasmodium* in their ecological and evolutionary context – is particularly important for our understanding of malaria transmission and how to control it. Indeed, describing the processes that create and maintain variation in mosquito immune responses and parasite virulence in natural populations may be as important to this endeavor as describing the immune responses themselves. For historical reasons, Ecological Immunology still largely relies on studies based on non-natural model systems. There are many reasons why current research should favour studies conducted closer to the field and more realistic experimental systems whenever possible. As a result, a number of researchers have raised concerns over the use of artificial host-parasite associations to generate inferences about population-level processes. Here I discuss and review several lines of evidence that, I believe, best illustrate and summarize the limitations of inferences generated using non-natural model systems.

Key words: *Anopheles gambiae*, *Plasmodium falciparum*, mosquito-malaria interactions, model systems, inferences, ecological immunology, micro-coevolution, macro-coevolution life history trade-offs, transcriptomes.

INTRODUCTION

The majority of malaria-associated human mortality is caused by *Plasmodium falciparum*, a haemosporidian parasite of man that occurs in most tropical and sub-tropical regions of the world and is transmitted by anopheline mosquito species. It has long been recognized that understanding the mosquito immune response to infection by *P. falciparum* may be the key to finding ways of interrupting its transmission. Thus parasitologists and, more recently, insect molecular biologists have focused their research on mosquito immune-related genes and particularly on genes responsible for mosquito refractoriness to the malaria parasite, as well as on parasite-induced fitness costs to mosquito vectors. Since experimental infections of humans or primates are usually not possible much of that research is based on vertebrate/mosquito/*Plasmodium* model-systems which, as will be discussed in the next sections, often involve mosquito/*Plasmodium* associations that do not occur in nature.

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Ecological immunology is a relatively new discipline that focuses on the mechanism and function of immune responses in their ecological context. Since its focus is on population level processes, ecological immunology uses naturally-occurring host-parasite association as model systems. When it comes to research on mosquito/*Plasmodium* interactions, ecological immunology investigates how ecological factors affect interactions between mosquito vectors and the malaria parasite to create and maintain variation in host immune responses and *Plasmodium* virulence in natural populations. However, in contrast to what is commonly accepted in Ecological Immunology, when it comes to malaria research, studies of mosquito/*Plasmodium* interactions still largely rely on the non-natural model-systems used in the past. This paradox forms the basis of the ongoing controversy on the use of model-systems for ecological immunological studies of mosquito/malaria interactions and can be explained by the discipline's dual origins in Evolutionary Ecology and Parasitology.

EVOLUTIONARY ECOLOGY IN THE LATE 1980s–1990s

In order to understand the foundation of ecological immunology one needs to take a closer look at

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fundamental research in ecology in the late 1980s and 1990s. This period of time saw important transitions in a research discipline that had grown increasingly intolerant of classic observational studies and advocated instead experimentation as the only rigorous way for testing causal relationships between natural processes. Epitomized by the work of Richard Dawkins (Dawkins, 1976), Bill Hamilton (Hamilton, 1964*a*, *b*) and others, evolutionary thought had progressively permeated ecology's every topic, effectively bridging it with what was previously known as evolutionary biology whilst paving the way for evolutionary ecology. The study of host–parasite interactions is a particularly dynamic and fast-expanding field of research focusing on topics as diverse as the evolution of parasite virulence (Ewald, 1983; May and Anderson, 1983; Ebert and Herre, 1996), parasite local adaptation (Ebert, 1994; Gandon *et al*. 1996), and the role of parasites in sexual selection and the evolution of ornaments (Hamilton and Zuk, 1982; Andersson, 1986; Folstad and Karter, 1992). In order to maximize the strength of inferences, the emphasis is deliberately placed on field experiments of natural hosts and parasite populations, as well as on the thoroughness of experimental design and analysis. Parasite-imposed fitness costs are also studied in relation to host life-history trade-offs, such as those between current reproduction and future reproduction or survival (Forbes, 1993; Perrin, Christe and Richner, 1996; Richner and Tripet, 1999). Given their underlying importance in relation to most of the above-mentioned topics, host immune defences received increasing attention and by the mid-nineties, there were enough research groups measuring and manipulating body condition and immune responses in natural host-parasite associations (Lochmiller, Vestey and Boren, 1993; Moller and Saino, 1994; Deerenberg *et al*. 1997) to warrant a review on that emerging discipline. 'Ecological Immunology: costly parasite defenses and trade-offs in Evolutionary Ecology' (Sheldon and Verhulst, 1996) focuses almost entirely on vertebrate host-parasite associations with the exception of a single study on bumble bees (Konig and Schmid-Hempel, 1995). However, in the next few years insect ecological immunology began to establish itself as a field of research in its own right (Kraaijeveld and Godfray, 1997; Schmid-Hempel and Schmid-Hempel, 1998; Siva-Jothy, Tsubaki and Hooper, 1998; Kurtz, 2000; Rolff and Siva-Jothy, 2003).

THE PRAGMATIC HERITAGE OF PARASITOLOGY

In stark contrast to evolutionary ecology, parasitology's applied research emphasizes practicality and feasibility, and often relies on non-natural host-parasite model systems to study the life-stages of parasites inside their hosts. Given the impracticality of experimentally infecting primates, studies dating as early as the 1930s compared the permissiveness of various mosquito species to bird malaria first (Huff, 1927), and later rodent malarias in order to find an alternative model system to human malaria (Garnham, 1963; Killick-Kendrick and Peters, 1978). Ideal mosquito-malaria associations were those in which the malaria parasite completed its entire cycle in a manner comparable to what was known of *P. falciparum* in its natural vectors (Garnham, 1963; Killick-Kendrick and Peters, 1978). The observed difference in permissiveness between vector species was first considered to be the key to understanding mosquito-malaria interactions; until accumulating evidence that variation in resistance also occurred between individuals within species led researchers to drop that avenue of research altogether (Huff, 1935). Eventually, a limited number of model systems established themselves: the avian malaria-based systems *Aedes aegypti*/*Plasmodium gallinaceum* and *Culex pipiens fatigans*/*P. cathermerium*; and several associations based on rodent malarias, *Anopheles gambiae* and *An. stephensi*/*Plasmodium berghei* and *P. yoelii* and *An. stephensi*/*P. chabaudi* (Garnham, 1963; Yoeli *et al*. 1965; Killick-Kendrick and Peters, 1978). For a long time, model systems involving these unnatural mosquito/*Plasmodium* associations were the only option for experimental studies of mosquito/*Plasmodium* interactions and they make for the vast majority of the current literature on mosquito/malaria interactions (Tripet, Aboagye-Antwi and Hurd, 2008). The positive side of that heritage is that we know quite a lot about the development of *Plasmodium* in the mosquito and about the mosquito immune response. The downside, of course, is that much of our current understanding of mosquito-*Plasmodium* interactions stems from non-natural model systems and remains to be validated using natural associations. This should pave the way for research on the processes that create and maintain variation in mosquito immune responses and *Plasmodium* development in natural populations (Boete, 2005; Tripet *et al*. 2008).

MOSQUITO-*PLASMODIUM* MODEL SYSTEMS AND THEIR INFERENCES

One can see how the focus of evolutionary biologists on fundamental research, their evolutionary considerations and awareness of experimental designs and the strength of inferences will drive them away from non-natural systems which – in their view – cannot generate inferences applicable to the 'real world' of natural populations. This view has to be contrasted with that of parasitologists who consider studying life-threatening diseases a priority that warrants the development of practical laboratory-based model systems in order to conduct applied research ultimately leading to disease control. Today's literature on the ecological immunology of

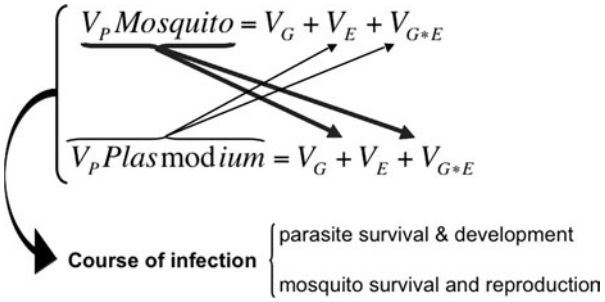

Fig. 1. Phenotypic variance components in infected mosquitoes (first equation) and in *Plasmodium* parasites (second equation). Where V_P is the phenotypic variance among individuals, V_G the genetic variance, V_E the variance due to the direct effect of the environment, and V_{G*E} the genotype-by-environment interaction, which occurs when the effect of the environment differs among genotypes. Thus, the phenotypic variance observed among hosts and parasites in natural populations is due to genetic and environmental factors as well as the interactions between the two. Both host and parasites strongly influence the course of infection and their respective phenotypic variances can be considered an essential component of each other's 'environment' that strongly affect their V_E and V_{G*E} terms.

mosquito/*Plasmodium* interactions reflects these contrasted heritages (Tripet *et al*. 2008). In the following sections, I will discuss several aspects of host-parasite interactions that clarify the limitations of non-natural model systems and emphasize the importance of ecological immunological studies based on natural associations.

Host-parasite interactions as a complex phenotype

In the natural environment, the outcome of mosquito infections with *Plasmodium* gametocytes are particularly complex phenotypes (Fig. 1). In simple quantitative genetics terms the variation observed in such phenotypes can be summarized by two equations: The first one describing the phenotypic variation in the infected mosquitoes (e.g. survival, immune response level and body condition) which is determined by variation in mosquito genetic and environmental factors, and interactions between those factors; and the second one describing the phenotypic variance of the infecting *Plasmodium* parasites (survival, multiplication, rate of development, etc.) which is determined by variation in parasite genetic and environmental factors and interactions between those factors (Tripet *et al*. 2008). Both host and parasites equations strongly influence the outcome of infections and their respective phenotypic variances can be considered an essential component of each other's 'environment' (Fig. 1). The importance of mosquito and *Plasmodium* genetic and environmental factors on parasite development and virulence – i.e. parasite-induced fitness costs to the host in the form of decreased fertility or survival (see discussion below) – has been demonstrated using non-natural model-systems and in a few studies of natural associations (reviewed in Tripet *et al*. 2008).

One of the challenges of ecological immunology is to help understand how ecological factors affect mosquito/malaria interactions to create and maintain variation in mosquito immune defence and *Plasmodium* virulence in natural populations. In this regard, laboratory-based studies are constrained by a variety of factors that tend to decrease phenotypic variation among individuals through various selection pressures (Table 1). This is true even in laboratory studies of natural mosquito/*Plasmodium* associations whose inferences should be more easily extrapolated to natural populations than those from non-natural associations. Generally, the lack of phenotypic variation in mosquitoes and parasites that characterizes laboratory experiments limits our ability to study the whole palette of host-parasite interactions (Table 1). However, laboratory-based studies conducted far away from malaria endemic countries are not the only option available to researchers. Indeed, the increasing large number of facilities dedicated to malaria research in African countries makes it ever easier for studying mosquito-malaria interactions nearer to the field (Table 2).

Host-parasite coevolution and local adaptation

Coevolution between hosts and their parasites is commonly seen as an evolutionary arms race between populations of parasites that evolve to maximize their fitness, which depends on their successful transmission to new hosts, and population of hosts that evolve mechanisms to limit their own fitness loss to parasites (Price, 1980, 1984; Poulin, Morand and Skorping, 2000). In doing so, hosts exert selection pressures on their parasites and, reciprocally, parasites exert selection pressures on their host, something that can readily be understood in mosquito/*Plasmodium* association given how strongly they are expected to affect each other's fitness components (see Fig. 1). One must keep in mind that reciprocal selection also occurs between the vertebrate hosts and the asexual *Plasmodium* stages; hence malaria evolution is essentially an intimate, triangular affair between parasite, vector and vertebrate hosts. In this regard, it is worth remembering that human, rodent and bird malarias cannot develop in each other's vertebrate hosts thereby underlining crucial genetic differences between those parasites.

Detecting and quantifying host-parasite coevolution is not a simple task especially when parasite specificity is low. In parasites with high specificity, such as *Plasmodium*, coevolution translates in congruence between the host and parasite phylogenies in what is commonly know as phylogenetic tracking (Poulin, 2007). Recovering the macroevolutionary

Table 1. Common factors associated with laboratory-based experiments that negatively affect studies of mosquito-malaria interactions

Phenotypic Variance component†	Source of bias	Processes	Consequences
Mosquito genetic variance	Colonization	Selection for mating in cage Selection for oviposition in cage	Limited number of strains available with limited genetic variation
	Laboratory conditions	Selection for lab-specific breeding scheme Inbreeding	
Mosquito environmental variance	Laboratory conditions	Lab-specific stable environmental conditions	Limited environmental variance
Mosquito genetic*environmental variance	Colonization and laboratory conditions	See above	Limited genetic* environmental variance
Plasmodium genetic variance	Isolation Cloning	Selection for *in vitro* culturing Intentional control of genetic background	Limited number of strains available with limited genetic variation
	Culturing	Selection for lab-specific culturing scheme	
	Mosquito infections	Selection for gametocyte infectivity	
Plasmodium environmental variance	Laboratory conditions	Lab-specific environmental conditions affecting mosquitoes (see above) and/or *Plasmodium*	Limited environmental variance
Plasmodium genetic* environmental variance	Isolation Cloning Culturing Mosquito infections Laboratory conditions	See above	Limited genetic* environmental variance

† Note that the importance of some of these factors has been tested experimentally (reviewed in (Tripet *et al*. 2008)).
* This corresponds to a multiplication sign (see Fig. 1).

Table 2. Commonly available options to study naturally-occurring interactions between *Anopheles gambiae* and *Plasmodium falciparum*

Experiments conducted with:	Advantages	Disadvantages
Naturally infected wild-caught blood-fed female mosquitoes	Natural genetic variation in mosquitoes and parasite	No control of mosquito age No control of previous infection Only feasible in areas highly endemic for malaria
Progeny of wild-caught blood-fed female mosquitoes fed infected blood from patient	Natural genetic variation in mosquitoes and parasite Control of age No previous infection	Only feasible in endemic malaria areas Only a fraction of individuals will feed on membranes
Colonized mosquitoes fed gametocytes produced *in vitro*	Feasible away from malaria endemic areas Full control of experimental conditions	Costly infrastructure Mosquitoes with little genetic variation and heavily selected (see Table 1) Parasites with little genetic variation and heavily selected (see Table 1)

history of *Plamodium* and other Haemosporidia has kept systematic parasitologists confused for decades and, until recently, there was still considerable debate on the most likely common ancestor of *P. falciparum* (Roy and Irimia, 2008). The most recent and complete analyses included sequence data from the nuclear, mitochondrial and plastid genomes (Perkins, Sakar and Carter, 2007; Martinsen, Perkins and Schall, 2008) and compared the distribution of rare indels in the rDNA and full mitochondrial genomes in a large number of Haemosporidia (Roy and Irimia, 2008). The results from both approaches do not support a common *P. falciparum* and rodent malaria clade but suggest instead

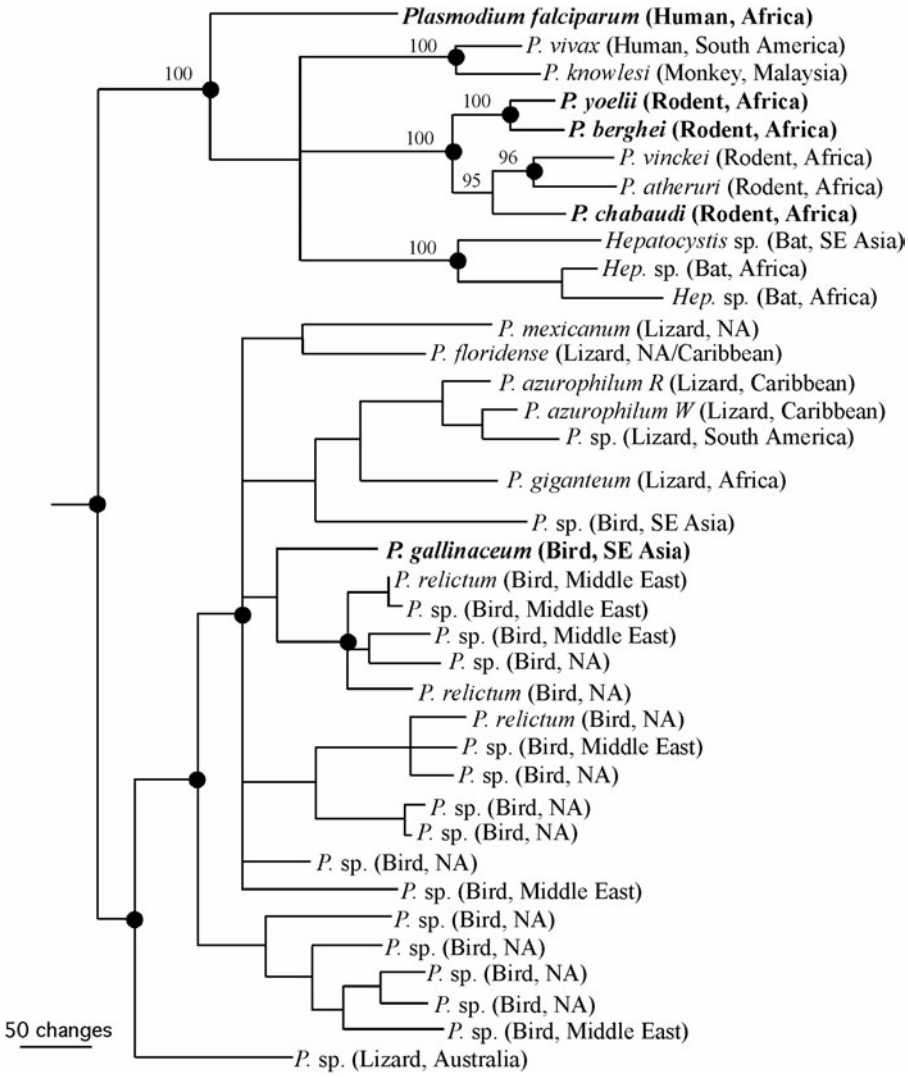

Fig. 2. Majority-rule consensus phylogram recovered using maximum parsimony and Bayesian analyses of four genes across the parasite three genomes (modified with permission from (Martinsen *et al*. 2008). Only part of the tree is presented here. Rodent and avian malarias commonly used in studies of mosquito/*Plasmodium* interactions are in bold. Dots on notes indicate $\geqslant$95% posterior probability support nodal support values estimated by Bayesian analysis. Parsimony bootstrap values greater or equal to 95% are also indicated. Taxon labels are the genus based on morphology seen in microscope blood smears, and species when that identification could be made with confidence. Vertebrate hosts and region of samples are indicated.

that they share a distant ancestor (Fig. 2) (Martinsen *et al*. 2008; Roy and Irimia, 2008). The hypothesis of a common origin with bird malaria (Waters, Higgins and McCutchan, 1991, 1993) is not supported either. *P. falciparum* sequence data is very distinct from those distant relatives. For example, there is an average 14·1% DNA sequence divergence (range 12·3–15·3%) between *P. falciparum* and any of the rodent malarias whilst the average level of divergence between all rodent malarias is 6·4% (Martinsen personal communication). *P. falciparum*'s closest relative would be the simian-human malaria parasite *P. reichenowi* (Roy and Irimia, 2008). Thus, these results provide evidence of some level of phylogenetic tracking between the phylogenies of *Plasmodium* and that of their vertebrate hosts. Using the mutation rate in the sequence of the small subunit ribosomal RNA and circumsporozoite surface protein genes as molecular clocks Escalante *et al*. (Escalante and Ayala, 1994, 1995) estimated that *P. falciparum* may have split from its simian ancestor $\sim$8–10 millions of years ago an event that may have coincided with the split between hominids and other great apes. Phylogenetic tracking between *Plasmodium* species and their insect vectors is more diffuse given that Plasmodium species are commonly transmitted by several mosquitoes species. However, evidence of phylogenetic tracking can still be detected at higher taxonomic levels. In their study, for example, Martinsen *et al*. (2008) show that all *Plasmodium* species fall within a single well-supported clade and that they are all transmitted by mosquitoes (Culicidae) apart from a single exception. Similarly, other genera of haemosporidia tended to form distinct clades each

transmitted by different Dipteran vector families (Martinsen *et al*. 2008).

Because parasites have typically shorter generation times and higher migration rates, and are under stronger selection pressure than their hosts, they are predicted to be adapted to their local host populations (Price, 1980; Gandon and Michalakis, 2002). Transplantation experiments are thus another approach to reveal coevolution, this time at the micro-evolutionary scale. Such studies compare the average parasite reproductive rate between combinations of sympatric and allopatric host and parasite populations. Local adaptation of parasites, which translates into higher reproductive rates of the parasite on sympatric hosts has been found only in some of the studies that tested for it (Ebert, 1994; Prugnolle *et al*. 2006). As shown by theoretical studies and a recent meta-analysis of all published studies on local adaptation, the likelihood that parasites will be more adapted to their local host depends on the migration rate of the parasite considered (Gandon and Michalakis, 2002; Greischar and Koskella, 2007).

Regardless of the direction of changes in parasite reproductive rate found in transplantation experiments, such significant difference between host and parasite populations within their species underline the importance of ongoing local coevolutionary processes. In natural mosquito/*Plasmodium* associations, recent experiments have showed evidence of local adaptation or differences between allopatric versus sympatric mosquito and *Plasmodium* populations in *An. gambiae* and *An. stephensi* infected with *P. falciparum*, and in *An. pseudopunctipennis* and *An. albimanus* infected with *P. vivax* (Hume *et al*. 2007; Joy *et al*. 2008).

Trade-offs between fitness and immune defences

Given the strong evidence for the occurrence of micro and macro-coevolution between *Plasmodium* and its hosts, it is not surprising that few of the non-natural mosquito/*Plasmodium* combinations examined by parasitologists have been found to complete their exogenous life stages in a natural association-like fashion. From an evolutionary biologist's point of view, the optimization of non-natural mosquito/*Plasmodium* model systems essentially gives up on coevolutionary considerations and is equivalent to creating artificial host-switches. Host switches, of course, do occur in nature; but they are essentially chance events often accompanied by increased virulence of parasites on their new permissive hosts (Ebert and Herre, 1996).

Due to the limited number of publications available on natural mosquito/*Plasmodium* interactions, quantifying differences between unnatural model systems versus natural associations is difficult. One aspect of infection for which such comparison has been possible are potential *Plasmodium*-induced fitness costs in terms of mosquito survival (Ferguson and Read, 2002). In a particularly interesting meta-analysis of the limited number of studies available on this topic, Ferguson and Read (2002) found a significant negative effect of *Plasmodium* on mosquito survival across the literature based on non-natural model systems but not in studies focusing on natural associations. These results confirm the expectation that artificial mosquito/*Plasmodium* model-systems may only crudely replicate natural interactions and may be associated with higher parasite virulence.

Molecular biology of mosquito immune responses

Thanks to whole-genome transcript analysis, molecular biologists working on mosquito immune responses now have powerful tools to quantitatively and qualitatively compare natural and non-natural associations. Consequently, they too have repeatedly emphasized the limitations of non-natural model-systems (Cohuet *et al*. 2006; Dong *et al*. 2006; Michel *et al*. 2006). For example, Dong and colleagues (2006) in a comparison of *An. gambiae*'s gene expression during invasion of the midgut with *P. falciparum* and *P. berghei*, found stark differences in the number of regulated genes. In *P. falciparum* infections, 471 genes corresponding to 3·4% of the transcriptome were regulated in contrast to *P. berghei* infections where no less than 1102 genes or 8·1% of the transcriptome changed (Dong *et al*. 2006). Importantly, studies looking at the function of individual immunity-related genes through reverse genetics found that only a fraction of those genes affected infections with human and rodent malaria in a comparable manner (Dong *et al*. 2006; Mendes *et al*. 2008). Immunity genes whose importance for human malaria has been validated through experimental studies of the natural mosquito/*Plasmodium* association are thought to be essential and ancestral components of the mosquito's immune's response architecture (Mendes *et al*. 2008).

CONCLUSIONS

Whilst artificial model-systems obviously must retain some basic characteristics of the natural associations of their host and parasite components, it is difficult to assess just how much can be learned from artificial model systems and how much will have to be re-visited using natural associations (Tripet *et al*. 2008). The potential severity of the problems associated with inferences generated through the study of non-natural parasite associations partially depends on the questions asked. Whilst we can reasonably assume that fundamental or ancestral-like physiological and developmental characteristics are shared between all vertebrate malarias, the same cannot be said of questions relating to population biology and micro-evolutionary processes. The problems and

concerns discussed above have been hinted at or voiced by an increasing number of researchers including evolutionary biologists, molecular biologists and parasitologists (Boete, 2005; Cohuet *et al*. 2006; Dong *et al*. 2006; Martinsen *et al*. 2008). Indeed, understanding how ecological factors affect mosquito/malaria interactions to create and maintain variation in mosquito immune defence and *Plasmodium* virulence in natural populations is generally recognized as paramount to our quest to resolve the malaria problem. However, recognizing that further advances in this field strongly depend on increasing our research effort based on studies of natural vector/*Plasmodium* associations whilst simultaneously minimizing the use of non-natural ones is still not fully accepted. It is hoped that the above discussion will serve as a strong incentive, for studying mosquito/*Plasmodium* interactions using *P. falciparum* and its natural vectors, and for doing so closer to the field.

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